REMARKS/ARGUMENTS

Claims 29 and 30 are pending. No claims are allowed.

Claim 30 was objected to under 35 USC 112, second paragraph. Claim 30 has been corrected. Reconsideration is requested.

Claims 29 and 30 have been amended to clearly recite that the antibodies are for use for a passive immunity vaccine as disclosed for instance at page 7, lines 21 to 30.

Claim 29 was rejected under 35 USC 103(a) as being unpatentable over Mora et al (Infect Immun. 1992 Aug; 60(8): 3442-5) in view of Liang et al (Infection and Immunity; 66(5) 1834-1838) or Marsh et al (JAVMA, 209: 1907-1913). Mora et al is a paper dealing with polyclonal antibodies against *Cryptosporidium parvum*, a disease unrelated to that caused by *Sarcocystis neurona* in horses. One skilled in the art could not equate this prior art antibody to the present invention. Liang et al describes the Sn16 and Sn30 proteins and states that "no inhibiting activity correlating to Sn30 was noted". This is not a direction in the reference to use antibodies to Sn30 in a vaccine. Marsh et al relates to

identifying *Sarcocystis neurona* in horses and not to production of a passive immunity vaccine. Thus one skilled in the art clearly could not have produced the claimed invention from the combination of references. Reconsideration is requested.

Claim 30 was rejected under 35 USC 103(a) as being unpatentable over Avarzed et al (Journal of Clinical microbiology, July 1998, p. 1835-1839, Vol. 36, No. 7) in view of Liang et al or Marsh et al. The secondary references have been discussed. Avarzed et al is even more remote from the claimed invention since it discusses monoclonal antibodies against *Babesia equi* a tick-borne protozoan disease in horses. The combination of these references does not produce the claimed passive immunity vaccine.

Enclosed is a copy of a draft paper by the inventors which shows that a mixture of 16 and 30 kDa monoclonal antibodies was very effective in a mouse model for *Sarcocystis neurona*. These results are unexpected in view of the prior art. Applicants can provide this data in a Declaration Under 37 CFR 1.132, if necessary. Polyclonal antibodies would be equally as effective.

Thus it is now believed that Claims 29 and 30 are in condition for allowance. Notice of Allowance is requested.

Respectfully,

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Enclosure: Paper by the inventors (Monoclonal IgG Antibody-mediated protection against *Sarcocystis neurona* infection)

Monoclonal IgG antibody-mediated protection against *Sarcocystis neurona* infection

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Abstract

In order to understand immune correlates of protection and to develop effective immunization strategies against EPM disease, it is important to know if antibodies can confer protection against *Sarcocystis neurona* infection. This study was conducted to test the hypothesis that IgG antibodies confer protection against *S. neurona* infection. A panel of IgG monoclonal antibodies (MAbs) was evaluated in tissue culture systems and by using Gamma-Interferon-gene-Knockout (IFN-$^{\gamma}$-KO) mice. Each of the MAbs tested formed immuno-fluorescence on the entire surface of *S. neurona* merozoites to at least a titer of 1:1250 and recognized 16 and 30KDa bands by Western-blotting. Each MAb diluted 1:25 in culture media reduced the attachment and invasion of *S. neurona* merozoites into equine dermal (ED) cells compared to controls. A mixture of all MAbs diluted to 1:25 or 1:50 in culture media almost completely inhibited attachment and invasion. Additionally, intraperitoneal injection of IFN-$^{\gamma}$-KO mice with the MAbs one day before and the day of challenge and on day 1 and 2-post challenge conferred a considerable level of protection against infection with sporocysts of *S. neurona* delivered orally. This MAbs treatment prolonged the mean time to death (MTD) of immunized compared to control groups of mice. Passive transfer of the MAbs before the time of challenge and later showed no adverse or toxic effects on either the mice or the ED cultured cells. We conclude that the IgG response can impede the establishment of infective *S. neurona* parasites in the mouse intestine and these MAbs are candidate molecules for immunotherapy of *S. neurona* infection in horses. The potential for use of individual antigens as protective immunogens in preventing *S. neurona* infection is raised

Key words: *Sarcocystis neurona*; IgG; monoclonal antibody; passive immunization; protection.

Introduction

Sarcocystis neurona has become recognized as an important causative agent of equine protozoal myeloencephalitis (EPM) (Dubey et al., 1991). EPM is the most common neurologic disease in horses in the Americas (Dubey et al., 2001). An estimated 60 to 89.3 % of horses are seropositive (Beth et al., 2003; Rossano et al., 2003). In addition to being a cause of serious discomfort to horses, EPM also has been associated with adverse neurological function and can be often fatal (Mackay et al., 1997). Recent information indicates that EPM should be taken more seriously, not only because of its increasing prevalence but also because of its devastating economic impact on the horse industry in the United States (NAHMS, 1998; 2000). In spite of research efforts to develop therapeutic agents and vaccines against the causative agent of EPM, effective control remains elusive. No commercially available anti-parasitic chemotherapy is consistently effective in treating this disease. Likewise, the current vaccine strategy focuses on targeting the whole parasite antigen resulting in variable efficacy probably because immune serum produced varied greatly in the composition, isotype, and specificity of microbe-binding antibodies. In contrast, monoclonal antibodies (MAbs) provide a homogenous preparation with which to investigate the variables that contribute to antibody-mediated protection. Therefore, MAbs might be a potential alternative immunotherapy against *S. neurona* infection.

In the last decade, it has become increasingly evident that multiple independent mechanisms are involved in the development of *S. neurona* infection, depending on host and parasite factors, or a combination of both. However, the cellular mechanisms of pathogenesis of *S. neurona* are not well defined. Moreover, the significance of the antibody response to *S. neurona* infection is not clearly defined except for a few studies (Witonsky et al., 2004). The life cycle of *S. neurona* in horses is complex and involves asexual development within the horse neural tissues "aberrant intermediate host" as well as sexual development within the gut of the definitive host, the opossum. Infection of horses occurs via ingestion of food or water contaminated with the sporocyst stage of the parasite, followed by sporocyst excystation and liberation of sporozoites in the horse small intestine. This is followed by the crucial step of sporozoite invasion of horse gut epithelial cells into the blood stream, which is a key step prior to successful development and establishment of the infection in the neural tissues. For this reason, the interaction between parasite and host cells at this site plays a critical role as the first host

defense barrier and may provide promising molecular targets for vaccine intervention. Additionally, the merozoites remain in the extracellular phase till reach to the neural tissue during which they are susceptible to humoral immunity.

The role of antibody in protection against a microorganism is often studied directly, by passive antibody transfer in a relevant animal model. Our hypothesis was to test the potential for intraperitoneal inoculation of a mixture of MAbs to conferr protective immunity to *S. neurona* infection in IFN-$^{\gamma}$-KO mice. Using this mouse model, we have tested a panel of MAbs to determine the possible role of IgG antibody for humoral immunity to *S. neurona* infection. Our data showed that passive transfer of IgG MAbs can confer remarkable protection against exposure to *S. neurona* infection in tissue culture system and if parasite infection occurs in IFN-$^{\gamma}$-KO mice, their presence can ameliorate the onset and subsequent pathogenic manifestations of *S. neurona* infection even without synergistc T-cell help.

Materials and methods

Animals, animal room, and housing

Seven-to eight-week old inbred specific-pathogen-free female mice were used for the protection experiment. These mice (C.129S7 (B6)-*Ifng*^{tm1ts}) lack the functional gamma interferon gene (IFN-$^{\gamma}$-knockout) and have been widely used as a model for pathogenesis and infectivity studies of *Sarcocystis neurona*. Mice were obtained from a commercial breeder (Jackson Laboratories, Bar Harbor, Maine, USA). All described procedures were approved by the Institutional Animal Care and Use Committee and conducted in compliance with accepted standards of the *Guideline for the Care and Use of Laboratory Animals* (NIH Publication 86-23, revised 1996). The mice had been monitored by the vendor and found free of the following pathogens; *Klebsiella* species, cilia-associated respiratory bacillus, *Pasteurella pneumotropica*, *Staphylococcus aureus*, *Bordetella bronchiseptca*, *Corynebacterium kutscheri*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Mycoplasma* species, *Citrobacter rodentium*, *Pseudomonas aeruginosa*, *Salmonella* species, *Clostridium piliforme*, *Helicobacter* species, *pneumocystis carnii*, ectromelia virus, mouse hepatitis virus, mouse parvovirus, mouse rotavirus, mouse encephalomyelitis virus, K virus, Hantaan virus, lactate dehydrogenase-elevating virus, mouse minute virus, pneumonia virus of mice, sendani virus, lymphocytic choriomenengitis virus, polyoma virus, thymic virus, mouse adenovirus, mouse cytomegalovirus. Also, mice were

examined every day for signs of illness and monitored by periodic tape tests for ectoparasites and fecal flotation for endoparasites and results were negative.

Mice were maintained and received humane care in a specific pathogen-free (P-2 level) animal facility at the Michigan State University Laboratory Animal Containment Facility. Animals were housed, three to five mice per cage, in autoclaved 11.5x7.5x5-in. polycarbonate cages with stainless steel lids and covers fitted with a 0.22-μm filter (micro-Isolator™, Lab Products, Inc., Maywood, N.J.). Maple hardwood chip bedding (Harlan Sani-Chips, Harlan TeKlad, Madison, wis.) was changed twice weekly. Animals were maintained on a laminar flow bench with controlled temperature (22±2°C), humidity (60± 10%), and lighting (12-h light/dark cycle). A commercially formulated irridiated mouse diet (7913; Harlan TEKIAD, Madison, wis.) and acidified water (pH 2.5 to 3.0) were offered *ad libitum*. The floor of the animal room was cleaned by use of a vacuum cleaner, and the racks of the laminar flow benches and the floor were disinfected with a 50 ppm solution of sodium hypochlorite. Mice were acclimatized for at least one week prior to being used in the experiment.

Parasite and tissue cultures

For the in vitro experiments, *S. neurona* isolate MIH1 was used. Merozoites of this strain were cultured from neural tissue of an EPM diseased horse from Michigan, USA in 1997, and thereafter maintained in our laboratory in equine dermal (ED) cell cultures (Mansfield et al., 2001). ED cells; the cell line used throughout this study were purchased from American Type Culture Collection (ATCC, CRL-1390) and used between passages 25-29. Cells were routinely grown in 75-cm^2 cell culture flasks in 15-20 ml of Dulbecco modified Eagle's medium (DMEM) supplemented with 10 mM HEPES buffer, L-glutamine, 10,000 IU/ml penicillin G sodium, 10,000 μg/ml Amikacin, 250 μg/ml Amphotericin B, nonessential amino acids, 100 mM sodium pyruvate, and 10% heat inactivated fetal bovine serum (FBS) (GIBCO Invitrogen Corp., Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO_2/95% air until the monolayer became confluent when the medium was changed to maintenance medium (6% FBS). This medium supported both host cells and parasites. The medium was changed once a week. ED cells were passaged once a week.

For the in vivo experiment, *S. neurona* sporocysts were originally isolated from an

opossum gut in 2002 and purified by centrifugation of the mucosal homogenate on potassium bromide discontinuous density gradient centrifugation as described (Elsheikha et al., 2003). Sporocysts were genotyped using the diagnostic 25/396 DNA marker as described (Tanhauser et al., 1999). In March 2003, ~ 1,000 of these sporocysts induced neurological signs and encephalitis in an inoculated IFN-$^{\gamma}$-knockout mouse 33 days post inoculation (DPI). One week prior to mice inoculation, sporocyst viability was assessed by propidium iodide (PI) exclusion assay as described (Elsheikha and Mansfield, 2004a).

Monoclonal antibodies

We need to write some information about the source and nature of these Mabs obtained from IDEXX Company.

Evaluation of MAb Cytotoxicity

ED cells were dislodged from a confluent ED monolayer in a 75-cm^2 flask by 0.25% trypsin dissociation. Cell viability was tested by trypan blue dye exclusion assay and then plated in 24-well cell culture plates (Cat. No. 3526, Costar, Corning Inc., Corning, NY, USA) at ~ 2 X 10^3 cells per well and grown on 10% DMEM until confluent. Media was removed and cells were incubated with about 50 µl of the MAb (1:25 dilution in DMEM) for the same mount of time (1.5 hr) the merozoites were exposed to the MAb in the in vitro parasite inhibition experiment. Cells fed medium without MAb treatment were used as controls. One ml 6% FBS DMEM was added to each well after 1.5 hr. After 24 hours, media was removed and replaced with fresh 6% FBS media.

Qualitative evaluation

MAb-treated and control cells were examined at 0 hr, 4hr, 8hr, 24hr, 48hr, and 96hrs, post treatment. The cell monolayers were inspected visually first at 200x magnification using an inverted microscope (Nikon, Olympus-CK2, Japan) for any signs of cell lyses such as the presence of rounded cells, detached cells, and/or granules. Then cells were examined at 400x magnification for the presence of intracytoplasmic vacuoles indicative of cytotoxicity. Experiments were run in triplicate.

Quantitative evaluation

In addition to microscopic observation, we used a quantitative assay to assess cytotoxicity of the MAbs. This was a non-radioactive cell proliferation technique known as the Methylene blue staining assay. This assay is based on the release of methylene blue from stained ED host cells, as reported for fibroblast cells (Oliver et al., 1989). Culture media was removed from the wells and cells were washed 3 times briefly with 1 ml of 0.12 M saline, and excess fluid was removed. The cell monolayers were then fixed by adding 1 ml of a 10% formaldehyde saline solution and the plate was incubated at room temperature (RT) for 30 min. The formaldehyde solution was removed into a separate hazardous waste container. One ml of filtered 1% (w/v) methylene blue stain was added and the plate was incubated for 30 min at RT. The dye was removed and wells were washed 4 times with 0.01 M 1x borate buffer (pH, 8.5). The cell bound dye was released by addition of 1 ml of elution solvent solution 1:1 (v/v) ethanol and 0.1 M HCl. The remaining cell monolayer stained with methylene blue and was checked microscopically. One ml of the eluted fluid per well was distributed into 10 wells of a 96-well plate. The absorbance of the contents of the wells was measured spectrophotometrically at 630 nm in a microplate reader (Bio-Rad) after correcting for background absorbance with wells containing only 100 µl of the elution solvent. This experiment was performed at 1, 2, and 4-days post treatment. Data were expressed as mean absorbance values (optical density) derived from 10 replicate samples in two separate experiments.

In vitro inhibition of parasite growth

ED cells were subcultured in 6-well plates (Cat. no. 3516, Costar) seeded with ~8×10^3 cells per well. For experimentation, cell monolayers were grown overnight or until confluent at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS. Prior to inoculation of plate wells with the parasite, merozoites were incubated in triplicate in a medium containing MAb at 37°C for 1.5 hr. Positive and negative assay controls were untreated merozoites and culture media, respectively. Plates were incubated in a sterile condition at 37°C in a 5% CO_2 atmosphere. Media was changed at 24hr and then weekly thereafter. In the first experiment, each MAb was tested individually at a dilution of 1:25 in 6% FBS DMEM. The numbers of plaques were

counted in all wells at 3 and 4-weeks post infection. In a second experiment, a mixture of all the MAb at dilution of 1:25 each and 1:50 each in 6% FBS DMEM were tested in triplicate as per individual MAb procedures. The numbers of plaques were counted in all wells at 3, 4, and 5-week post infection. Plaques were defined as areas where cells were coming loose surrounded by rounded up cells associated with the presence of merozoites and schizonts. In the first experiment, culture media containing merozoites were centrifuged for 10 min at 1,200 rpm to pellet the whole cells and large particles. Supernatants were removed and centrifuged for 30 min at 1,200 rpm. The pellets were then resuspended in 1.5-ml 6% DMEM which was aliquted into the required numbers of tubes. In the second experiment, *S. neurona* merozoites used were first separated from host cells using PD-10 column as described (Elsheikha et al., 2004b).

Measurement of Mabs Activity

Immunofluorescence assay (IFA)

For preparation of IFA slides, merozoites were harvested and washed with 1x phosphate buffered saline (PBS), and resuspended in PBS. Serial dilutions of the merozoite stock were placed in duplicate in wells of 12 well slides (20µl/well), air dried and fixed in acetone. Cerebrospinal fluid (CSF) from a horse previously tested positive for *S. neurona* antibodies was placed on each well and incubated for 30 min at 37°C. The slide was washed several times with PBS and then incubated with Fluorescein isothiocyanate- (FITC)- labeled goat anti-horse IgG- h+1 secondary antibodies in PBS and 0.1% Evans blue and incubated at 37°C for an additional 30 min. The slide was washed with PBS, cover-slipped and the optimum dilution of merozoites for IFA slides determined. The stock merozoite solution was diluted with PBS to the optimum and multiple slides prepared through the fixation step and stored frozen at -20°C until used.

Monoclonal antibodies (6A, 6B, 9A, 11A, 17A, 19B) were applied at 1:2500 and 1:10,000 dilutions in PBS and incubated for 30 min at 37°C followed by washing as above. Serum from a knockout mouse previously infected with *S. neurona* was used as a positive control. FITC-labeled goat-anti-mouse IgG-h+1 secondary antibody (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD, USA) diluted 1:10 in PBS and 0.1% Evans blue dye (as a general protein counterstain) were applied. Stained merozoites were observed by epifluorescent

microscopy using the SPOT RT slider "F" mount camera (model No. 2.3.1, Diagnostic Instruments Inc, Sterling Heights, MI, USA) and SPOT RT software V3.3. Pictures were taken using laser scanning microscope (LSM Zeiss axioskop 2 MOT, Jena, Germany) equipped with Zeiss LSM 5 Pascal Confocal Unit and LSM software version 3.0.

SDS-PAGE and western blotting

Parasite antigens and pre-stained protein molecular weight standards (Life Technologies, Inc., Gaithersburg, MD, USA) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using 12-18% continuous gradient gels under reducing conditions, and transferred on nitrocellulose membranes (Millipore). To block non-specific binding sites, the membrane was incubated for 60 min with Tween Tris Buffered Saline (TTBS) with Bovine Serum Albumin (BSA). Blots were incubated with (dil 1:10) primary MAbs in 1% BSA in Tween Tris (hydroxymethyl) aminomethane Buffered saline (B-TTBS) overnight. Then, blots were 3 x washed with TTBS, followed by 4 hr incubation with biolabelled goat antibody to mouse IgG (dil 1:10) antibody conjugated to HRP (KPL). To assess non-specific reactions, control strips were similarly prepared, but without using the primary antibody. Protein binding was detected by incubation of the blot for 45 min with ExtrAvidin peroxidase conjugate (Sigma) 1 µl/ml TTBS.

Preparation and injection of MAbs into mice

Six anti-*S. neurona* IgG MAbs (6A, 6B, 9A, 11A, 17A, 19B) were combined and evaluated for their prophylactic effect against a *S. neurona* sporocyst challenge in seven-to eight-week old IFN-$^{\gamma}$-KO mice. The rationale for selection of MAb combinations was based on preliminary data obtained from in vitro MAb binding inhibition assays. The ascites of 6A, 6B, 9A, 11A, 17A, and 19B were prepared for injection. A mixture of these antibodies was prepared from equal volumes of the ascitis fluids by diluting the fluids in sterile PBS such that each antibody will have a final dilution of 1:50. MAbs were administered intraperitoneally by injection of 0.5 ml of ascitis dilution. In the first experiment, mice were divided into 4 groups each of 3 mice as follow: (i) Group of 3 mice were gavaged with 500 sporocysts in 0.2 ml PBS per mouse intragastrically by using a rubber French catheter. One day prior to challenge and the day of challenge, and on the

first and second day post-challenge mice received combinations of ascites containing the six IgG MAbs by i.p. injection, (ii) Group of 3 mice were gavaged with 500 sporocysts and 0.5 ml of sterile 1x PBS I.P. and used as a positive control, (iii) Group of 3 mice were gavaged with 0.2 ml PBS and received 0.5 ml MAb ascites I.P. and served as a control for any potential side effect of the antibodies, (iv) Group of 3 mice were gavaged with 0.2 ml PBS and received 0.5 ml of sterile 1x PBS I.P and served as a negative control. This experiment was repeated again in a second trial using the same design used in the first experiment with the following two modifications: (1) the number of mice was increased to 5 mice per group and (2) the inoculation dose was lowered to 100 viable sporocysts per mouse.

Documentation of *S. neurona* infection in mice

Mice were monitored every day for the development of any clinical signs. As soon as mice showed any clinical signs of illness, they were euthanized by CO_2 inhalation. To determine if the mice that survived the challenge possessed the same parasite burden in their brains and other body organs, all remaining mice and control mice were killed at the same time the positive control, untreated mice were killed. Specimens from lung, intestine, liver, spleen, kidney, heart, and muscles were collected, fixed in 10% neutral buffered formalin, and processed for histopathology and immunohistochemistry. Also, the brains were halved by sagittal section, and small pieces of forebrain, midbrain, and cerebellum from each of the halves were examined by immunohistochemistry and IFA. The presence of schizonts or merozoites was scored histologically as previously described (Dubey and Lindsay, 1998).

Portions of the brain were used for extraction of the parasite DNA for confirmation of the infection by PCR and sequence analyses. The brain of each mouse was assayed for the presence of the schizont and merozoite stages of the parasite by inoculation into confluent monolayer of ED cell cultures in T25-cm flasks of brain homogenate in DMEM as described (Dubey and Lindsay, 1998). All inoculated culture flasks were monitored daily for the appearance of any signs of parasite growth and plaque formation under a light inverted microscope (Carl Zeiss, Opton, Columbia, MD, USA).

To test for the potential for excretion of sporocysts in mice feces following challenge with *S. neurona* sporocysts, feces from the animal bedding were collected daily for the first 3 days post inoculation and examined for the presence of sporocysts using the sucrose fecal

floatation technique, Diamant-Fuchsin stain, and PCR-sequence analyses as described (Elsheikha et al., 2004a).

Statistics

Results were reported as means plus or minus standard errors of the mean. Differences between groups (MAb-treatment and controls) in all experiments were initially explored for significance using the Fisher's Exact Test. The Fisher exact probability test is a non-parametric technique for analyzing discrete data when the two independent samples are small in size. It is used when the scores from two independent random samples all fall into one or the other of two mutually exclusive classes, in this study either death or survival of mice. This analysis was followed by examination by one-way analysis of variance (ANOVA). Calculations were performed using commercially available statistical software (SAS PC Version 8, SAS Institute Inc., Cary, North Carolina). A P value less than 0·05 denoted statistical significance.

Results

MAbs toxicity on cultured host cells

Following MAbs treatment, the in vitro culture system used allowed us to study any specific cytologic changes to ED cells as well as to quantify any cytotoxicity to the cells. Toxic effects of the MAb on cultured ED cells were evaluated by using microscopic observation. The viability of cell preparations was determined before the experiments using trypan blue dye exclusion assay and was found to be ~99%. In one plate, 2 duplicate wells were incubated for 90 min with the MAbs mixture, and then fresh media was added. In 2 wells of another plate, cells were not exposed to MAbs. In both cases, wells were inspected every four hr over a course of 96 hr to assess viability, integrity, and to examine any toxic effects of MAbs on the cells compared to control untreated cells. The condition of cells throughout the incubation period was monitored by Nikon phase-contrast microscopy. No significant difference was noticed between MAbs treated and untreated ED cells. MAbs used in this study did not cause any cytopatheic lesions in ED cells compared to controls. The cytotoxic effect of MAbs was also evaluated quantitatively using the methylene blue staining assay. This assay was used to determine if the MAbs had any toxic

effect on the host cells, which in turn would inhibit their metabolic activity and thus diminish their ability to multiply. This assay assesses the viability of the cells by comparing the numbers of viable-MAbs treated host cells to viable-untreated host cells. Cells treated with MAbs showed no measurable cytotoxicity over the course of 96 hr (Fig. 1).

MAbs inhibitory effect on *S. neurona* merozoites in vitro

Prior to in vivo passive transfer studies with MAbs, we studied the in vitro inhibitory characteristics of these antibodies alone, and in combination, against *S. neurona* infection in ED cell monolayers. Evaluation of the inhibitory effect of MAbs on parasite infectivity in tissue culture relied on microscopic observation of the host cell monolayers following infection with the parasites. The nature and extent of host cell damage were assessed using an inverted phase-contrast microscope and was scored for each treatment as the mean number of plaques created by replicating merozoites (±s.e.m). For experiment 1, ED cells were cultured to confluence in 6-well culture plates. *S. neurona* parasites (~ 4×10^3/well) were added to the confluent ED monolayers. At the same time, ED monolayers in separate 6-well plates were incubated with the same parasite concentration after incubation with separate MAb for 1.5 hr. There were 3 replicates of each for this experiment. Data were recorded at 3, 4, and 5 weeks post inoculation. In experiment 2, ED cell monolayers were inoculated with *S. neurona* parasites (4×10^3/well). At the same time ED monolayers in a separate 6-well plate were incubated with the same parasite concentration after incubation with a mixture of MAbs at 2 different dilutions (1:25 and 1:50) for 1.5 hr. There were 3 replicates each for this experiment. Data were recorded at 3 and 4 weeks post inoculation. All MAbs used in the in vitro studies conferred a high degree of protection against *S. neurona* infection and significantly reduced mean plaque scores compared to those of control ($P < 0.05$). From a functional perspective, the most intriguing observation is that the MAbs used in this research either individually (Fig. 2A) or in combination (Fig. 2B) were all capable of inhibiting the parasite invasion to cultured cells. Parasites treated with MAbs produced lesser numbers of plaques than untreated parasites, which underwent unrestricted proliferation leading to substantial cell disruption.

MAbs recognizes distinct parasite proteins by Western immunoblotting and IFA assays

A reproducible banding pattern was observed for parasite lysates in western immunoblotting. MAbs reacted largely against an antigenic fraction with a mol. Wt. of at 30,000, although it also had some slight reactivity against a 16,000 mol. Wt. antigen. MAbs to *S. neurona* cell membrane antigens was measured with the IFA assay utilizing *S. neurona* cultured-derived merozoites. IFA demonstrated antibody binding to the *S. neurona* merozoite surface.

Prophylactic effect of IgG MAbs on *S. neurona* infection in mice

To determine whether MAbs could protect naïve IFN-$^{\gamma}$-KO mice against *S. neurona* infection, passive immunization studies were carried out. The quantity of antibodies used in protection studies was based on a previous determination of the amount of antibody required to be reactive in the IFA assay. A combination of MAbs administered prophylactically to mice delayed the onset and severity of infection in treated mice compared to controls. In the first experiment, infected, untreated mice developed clinical signs at 14 DPI, 2 days earlier than the infected and treated mice. Also, clinical signs were less intense in treated mice compared to the untreated mice. However, at 25, 26, and 27 DPI one mouse of each group were euthanized due to the progression of the disease. In the second experiment, infected and untreated mice developed clinical signs at 13 DPI, 6 days earlier than the infected and treated mice. On 29 DPI all the five infected and untreated mice were euthanised due to progession of severe clinical signs. In contrast, one out of five of the infected and treated mice was euthanised at 30 DPI because of a severe head tilit, one mouse was found dead 33 DPI and the remaining mice were euthanised at the same day, 33 DPI due to progression of clinical signs. All control mice whether mock-inoculated with PBS or MAbs did not develop any clinical signs throughout the whole experiments.

Detection of *S. neurona* sporocysts in mice feces

Fecal flotation and Diamant-Fuchsin staining assays did not detect any *S. neurona* sporocysts in any mice feces from all different mice groups. However, PCR using LSM1 and LSM2 primers produce a PCR product from DNA extracted from feces collected from cage of MAb-treated mouse group only.

Discussion

The present study was conducted to test the ability of specific monoclonal antibodies to protect IFN-$^{\gamma}$-KO mice from a challenge with a *S. neurona* infection. The results demonstrated that MAbs to *S. neurona* major surface antigens delayed the onset and severity of *S. neurona* infection in IFN-$^{\gamma}$-KO mice, thus providing support for the role of the surface antigens in active immunity and the potential use of these proteins as protective immunogens.

Although cell-mediated immunity appears to be the major component of the host's defense mechanism against intracellular organisms, antibody-based immunity, particularly serum IgG antibodies, play at least some part in the host's response against many intracellular pathogens (Robbins et al., 1995). Studies with monoclonal antibodies have demonstrated passive protection for several microbes where experiments with polyclonal immune serum had provided negative or inconsistent results, including *Candida albicans* (Han and Cutler, 1995), *Cryptococcus neoformans* (Dromer et al., 1987), *Listeria monocytogenes* (Edelson et al., 1999), *Leishmania mexicana* (Anderson et al., 1983), and *T. gondii* (Sayles et al., 2000). For these pathogens, the identification of protective monoclonal antibodies established the precedent that antibody could be effective and dispelled the notion that humoral immunity was ineffective due to an inherent limitation in the activity of this arm of the immune system.

In this study, microscopic examination of ED cells allowed us to study the cytotoxicity of MAbs. Likewise, the methylene blue assay allowed us to assess the host cell damage by MAbs treatment in a convenient, easy procedure. Oliver et al. (1989) described the methylene blue staining assay for use on rat and human fibroblast and lung cells. The assay is quick, inexpensive, and particularly applicable to adhered cells. The proliferation assay is based on the cellular staining properties of methylene blue, which can then be released from the cell into solution by lowering the pH, and then quantitated by measuring absorbance. This method was very effective for measuring cell numbers because it measures stain incorporation by all the cells in the well, thereby eliminating cell-to-cell field variation that occurs in a solid phase immunohistochemical methodology and standard counting.

On the basis of the number of detectable antigens in the western blot analyses, 30KDa and 16KDa appear to be the most predominant targets of the humoral response to the parasite. The utility of MAbs in the control of *S. neurona* infection was tested by passive immunotherapy

studies with MAbs in IFN-$^{\gamma}$-knockout mouse model. Results presented here demonstrate that IgG MAbs can reduce infection by *S. neurona* parasite. While cellular immunity is required to overcome *S. neurona* infection immunocompetent hosts, IgG directed to neutralization-sensitive merozoite epitopes may have utility in passive immunization against *S. neurona* infection. The six IgG MAbs selected recognized surface P30 on merozoites and have been shown to decrease infection levels in vitro and in the mouse model, indicating that P30 appears to have an essential role in the invasion process of the parasite and contains neutralization-sensitive epitopes. Because P30 is conserved among geographically diverse *S. neurona* isolates (Elsheikha and Mansfield, 2004b), present in both infectious sporozoite and merozoite stages, and might contain neutralization-sensitive epitopes, it may be a biologically relevant antigen, which can be targeted for immunological intervention. Pretreatment of *S. neurona* merozoites with MAbs abolished the adhesion and invasion of parasites to cell monolayers. These findings suggest the presence of contact-dependent inhibitory effects (i.e., involvement of carbohydrate-containing molecules in these processes) of the MAbs used in the present study. Results of the present study support the relevance of P30 and suggest that IgG targeted to this antigen may be a functional immune response to *S. neurona* infection. The in vitro and in vivo procedures as reported here provide a model system for studying the pathogenicity of *S. neurona* in detail. The knowledge of *S. neurona*-host cell interactions will provide insight into the mechanisms of host cytopathogenicity and the pathobiochemistry of *S. neurona* infection.

The incomplete efficacy of passive MAbs against *S. neurona* in KO mice particularly in those mice gavaged with 500 viable sporocysts suggests that antibody-mediated protection might be independent of T cells and implied that other mechanism may be operative and high levels of inhibitory antibodies are required to achieve complete protection. This suggest that vaccine-induced inhibitory antibody, along with cellular immunity, could offer protection against *S. neurona* infection. Given our knowledge that IgG is one important component of protection, it is reasonable to continue to focus resources aimed at the design of *S. neurona* vaccine immunogens that will elicit potent inhibitory antibodies to the parasite. While we do not know if KO mice model accurately predict the precise quantitative levels of antibody needed to protect horses, the data begin to spot the light on the type of antibody response that could play a role in protection against EPM disease in horses.

In addition to the amount of antibody, immunoglobulin-related variables such as antibody

specificity, isotype, and idiotype can have profound effects on antibody protective efficacy. However, host-related variables can also determine the outcome of passive protection experiments. For example, the protective efficacy of passive antibody to *Salmonella enterica* serovar *Typhimurium* is dependent on the mouse strain used (Eisenstein et al., 1984). For some pathogens, the efficacy of passive antibody is dependent on the presence of intact cellular immunity (Yuan et al., 1997). Also, antibody efficacy can depend on the microbial strain used despite the presence of the target antigen (Mukherjee et al., 1995). The exact number of genotypes of *S. neurona* that occur in nature is unknown. To date we have identified two different genotypes (designated H1 and H2) among 5 *S. neurona* isolates (Elsheikha and Mansfield, 2004b). We have not found mixed infections in naturally infected horses and do not yet know whether cross-reactive immunity plays a role in nature. Nevertheless, epidemiological questions such as these are of clear interest for vaccine development and are currently being investigated.

In summary, these initial studies have shown that MAbs can bind to the merozoite stage of the parasite life cycle and significantly inhibit infection in vitro. Additionally, passive immunization of IFN-$^{\gamma}$-KO mice with these MAbs suggests that the immunodominant surface antigens are partially protective antigens and as such are primary candidate for use in a subunit vaccine against the *S. neurona* infection. These findings point the way toward their testing in horse trials for immunotherapy of *S. neurona* infection in horses.

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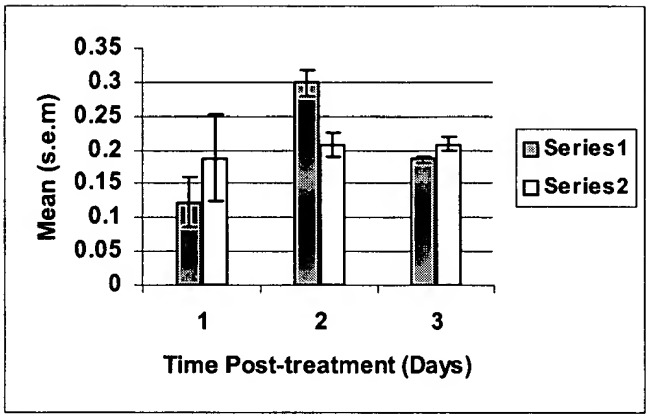
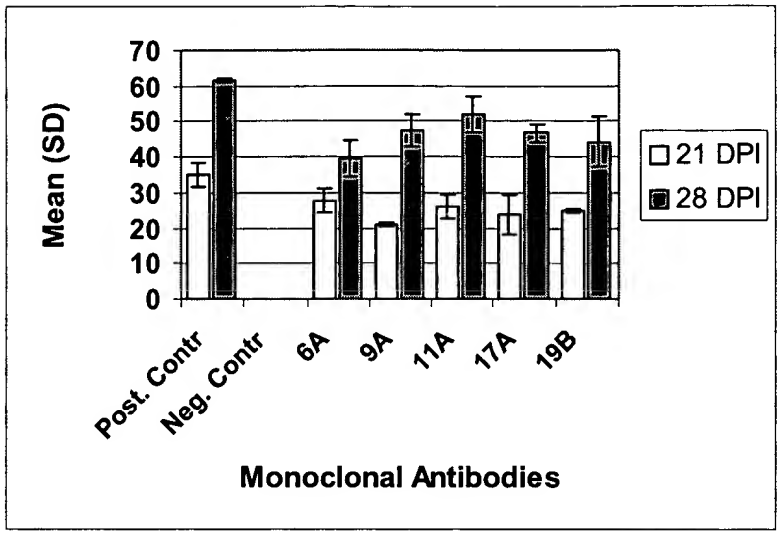


Figure 1. Time course of cytotoxicity of equine dermal cell monolayers by mixture of monoclonal antibodies (■) compared to control untreated cells (□). Cytotoxicity was determined by methylene blue assay as described in the text.



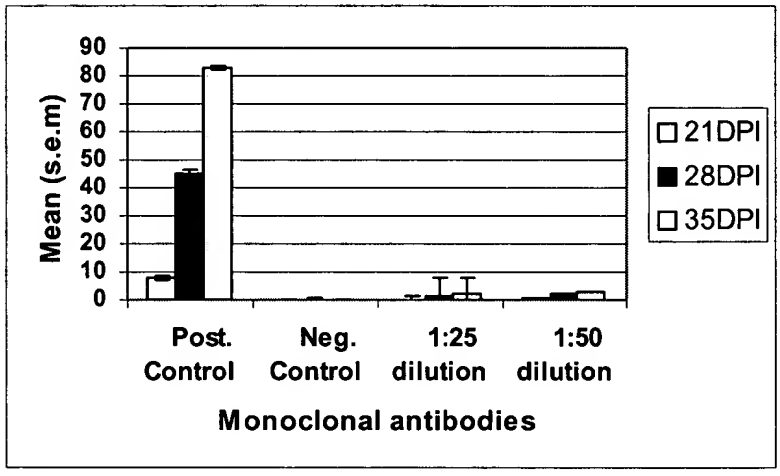


Figure 2. Inhibitory effect of monoclonal antibodies (MAb) against infection with *Sarcocystis neurona* merozoites in equine dermal cell cultures. (A) using individual MAb. (B) using MABs combination